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Involvement of the CD95 (APO-1/Fas) receptor/ligand system in drug-induced apoptosis in leukemia cells

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Cytotoxic drugs used in chemotherapy of leukemias and solid tumors cause apoptosis in target cells[1,2]. In lymphoid cells the CD95 (APO-1/Fas)/CD95 ligand (CD95-L) system is a key regulator of apoptosis[3–6]. Here we describe that doxorbicin induces apoptosis via the CD95/CD95-L system in human leukemia T-cell lines. Doxorubicin-induced apoptosis was completely blocked by inhibition of gene expression and protein synthesis. Also, doxorbicin strongly stimulated CD95-L messenger RNA expression *in vitro* at concentrations relevant for therapy *in vivo*. CEM and Jurkat cells resistant to CD95-mediated apoptosis were also resistant to doxorbicin-induced apoptosis. Furthermore, doxorbicin-induced apoptosis was inhibited by blocking F(ab')$_2$ anti-APO-1 (anti-CD95) antibody fragments. Expression of CD95-L mRNA and protein *in vitro* was also stimulated by other cytotoxic drugs such as methotrexate. The finding that apoptosis caused by anticancer drugs may be mediated via the CD95 system provides a new molecular insight into resistance and sensitivity toward chemotherapy in malignancies.

The mechanisms by which apoptosis is caused by anticancer drugs are not understood at the molecular level. The CD95/CD95-L system is a key regulator of apoptosis[3–6]. We have recently demonstrated that T-cell receptor (TCR)-triggered activation-induced cell death in peripheral T cells is mediated via CD95/CD95-L interaction[7]. Following TCR stimulation, T cells express CD95, a member of the tumor necrosis factor receptor/nerve growth factor receptor superfamily, which mediates apoptosis upon oligomerization[8–11] and produce CD95-L, a member of the corresponding family of TNF-related cytokines[7,12], which is found in a soluble or membrane-bound form[13,14]. CD95-L may cause autocrine suicide in sensitive CD95$^+$ T cells and fratricide or paracrine death in neighboring T cells[7]. We hypothesized that apoptosis induced by cytotoxic drugs at therapeutic concentrations in leukemia cells may involve the CD95 system. We used the prototype acute T leukemia (T-ALL) cell line CEM and doxorbicin, a drug used in chemotherapy of lymphoid leukemias. Induction of apoptosis by doxorbicin *in vitro* was found in a concentration range of 0.001 to 0.1 μg/ml (Fig. 1*a*). This corresponds to concentrations of doxorbicin measured *in vivo* in patient sera (0.001–0.02 μg/ml)[15,16]. Cell death at higher concentrations (1–10 μg/ml) was not always associated with typical DNA fragmentation (data not shown). To investigate whether doxorbicin-induced apoptosis depends on gene expression and protein synthesis, we treated CEM cells with cyclosporin A (CsA) or cycloheximide (CHX) in addition to doxorbicin (0.1 μg/ml). In the presence of CsA or CHX, doxorbicin-induced apoptosis (Fig. 1*b*) and DNA fragmentation (Fig. 1*c*) were completely inhibited[17,18]. This suggested that doxorbicin-induced apoptosis depends on gene expression and protein synthesis similar to activation-induced cell death in normal peripheral T cells. CD95 is expressed on most T-ALL cell lines such as CEM and Jurkat and on leukemic cells from patients[19]. We therefore studied doxorbicin-induced apoptosis in CEM and Jurkat cells sensitive or resistant (CEM^R, Jurkat^R) to CD95-mediated apoptosis (Fig. 2*a*). These cell lines were resistant to CD95-mediated apoptosis up to 1 μg/ml of the anti-CD95 antibody. In contrast, the parental cell lines CEM and Jurkat were sensitive to low concentrations of anti-CD95 antibody (0.01 μg/ml). When we compared doxorbicin sensitivity of these CD95-sensitive or resistant cells, a marked difference was found. In CEM^R cells the concentration of doxorbicin (the median effective dose, ED_{50}) necessary to induce apoptosis was more than 100 times that necessary to induce the same effect in parental CEM cells. Also in Jurkat^R a 30-fold increase in drug resistance was found. In addition, doxorbicin-resistant cells, generated through continuous culture in doxorbicin (up to 0.1 μg/ml), were found to be resistant to CD95-mediated apoptosis (data not shown). This suggested a requirement for an intact CD95 pathway in doxorbicin-induced apoptosis. We next investigated whether doxorbicin-induced apoptosis in CEM cells could be inhibited by blocking F(ab')$_2$ anti-APO-1 (anti-CD95) antibody fragments previously shown to lack agonistic function and to inhibit autocrine T-cell suicide[7]. Incubation of CEM cells with F(ab')$_2$ anti-APO-1 completely inhibited doxorbicin-induced apoptosis (Fig. 2*b*). This clearly indicated that apoptosis in CEM cells induced by therapeutic concentrations of doxorbicin was mediated via CD95/CD95-L interaction. We therefore asked whether drug treatment of CEM cells stimulates expression of CD95-L. Incubation of CEM cells with doxorbicin at concentrations from 0.001 to 0.1 μg/ml stimulated expression of CD95-L mRNA as shown by northern blot analysis (Fig. 2*c*) and quantitative reverse transcriptase polymerase chain reaction (RT-PCR) (Fig. 2*d*). Doxorubicin-induced CD95-L mRNA expression showed an early peak (at 4 hours) with a tenfold increase over baseline levels. We next investigated whether other cytotoxic drugs used in chemotherapy of leukemias such as methotrexate could also induce CD95-L mRNA expression in CEM cells. Methotrexate also stimulated expression of CD95-L in CEM cells at concentrations present *in vivo* during therapy (10–50 μg/ml) (Fig. 2*e*). However, methotrexate-stimulated CD95-L mRNA expression showed a peak at 24 hours corresponding to delayed kinetics in methotrexate-induced apoptosis in CEM cells (peak apoptosis at 48 hours, data not shown). A slight increase in levels of CD95-L mRNA was also found upon treatment of CEM cells with cytarabine, etoposide

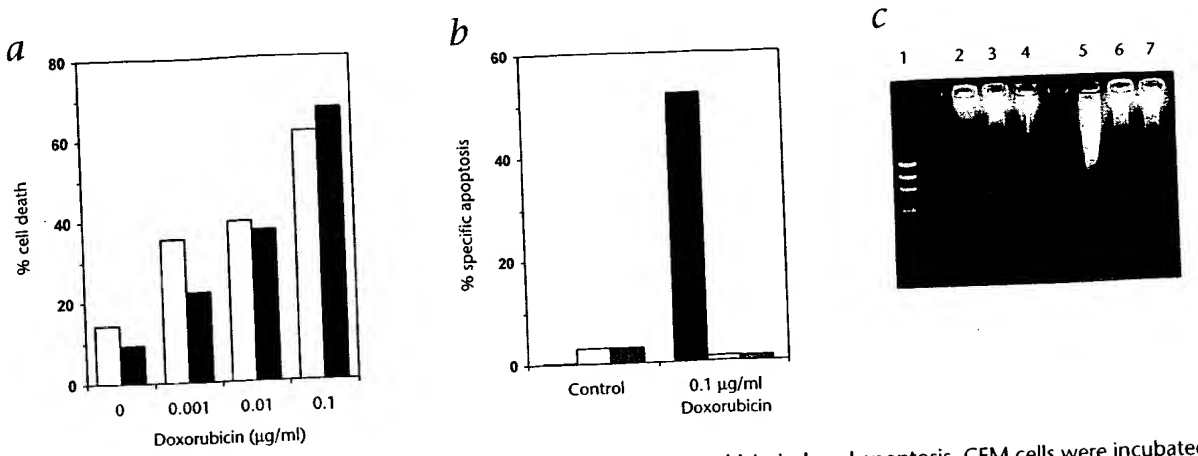

Fig. 1 Doxorubicin-induced apoptosis in CEM cells. *a*, Dose response of doxorbicin-induced apoptosis. CEM cells were incubated for 30 h in 96-well plates (5×10^4 cells/well) with doxorbicin at concentrations indicated. The percentage of dead cells was measured by forward/side scatter (FSC/SSC) analysis as described[24] (black bars) or by analysis of hypodiploid DNA (white bars). Data are given as mean of triplicates with a standard deviation of less than 10%. Similar results were obtained in three different experiments. *b*, Inhibition of doxorbicin-induced apoptosis by cyclosporin A (CsA) and cycloheximide (CHX). CEM cells (5×10^4 cells/well) were preincubated with medium (black bars) 0.1 μg/ml CHX (white bars) or 0.1 μg/ml CsA (grey bars) for 1 h followed by addition of 0.1 μg/ml doxorbicin. After 30 h apoptotic cells were measured by FSC/SSC analysis on a FACScan[24]. Percentage of specific cell death was calculated as follows: 100 × (experimental dead cells (%) — spontaneous dead cells in medium (%))/100% — spontaneous dead cells in medium (%)). Data are given as means of triplicates with a standard deviation of less than 10%. Similar results were obtained in three different experiments. *c*, Analysis of doxorbicin-induced DNA fragmentation pattern. CEM cells (2×10^7) were incubated for 48 h as indicated. Molecular weight marker (lane 1), cells incubated in medium (lane 2), cells treated with 0.1 μg/ml CsA (lane 3), 0.1 μg/ml CHX (lane 4), 0.1 μg/ml doxorbicin (lane 5), 0.1 μg/ml doxorbicin and pretreated with 0.1 μg/ml CsA (lane 6), 0.1 μg/ml doxorbicin and pretreated with 0.1 μg/ml CHX (lane 7).

and carboplatin (data not shown). To demonstrate CD95-L protein, western blot analysis of CEM cells was performed (Fig. 2*f*). A strong upregulation of baseline levels of CD95-L protein was observed after treatment of CEM cells with doxorbicin (8 hours) or methotrexate (24 hours).

These data demonstrate that apoptosis in leukemia cells triggered by cytotoxic drugs such as doxorbicin and methotrexate involves the CD95 system. Upon treatment with these drugs, CD95-L expression is induced that mediates apoptosis by fratricide or autocrine and paracrine death. Inhibition of CD95-L expression also inhibits doxorbicin-induced apoptosis. Thus, CsA blocks CD95-L expression and doxorbicin-induced apoptosis (Fig. 1*b* and data not shown). Furthermore in contrast to parental CEM cells, no doxorbicin-induced CD95-L expression is observed in doxorbicin-resistant CEM cells (data not shown). The concentrations at which this mechanism of toxicity is induced by doxorbicin *in vitro* (0.001–0.1 μg/ml) correspond to plasma concentrations measured in patients after continuous infusion or bolus injection of doxorbicin[15,16]. Recently, triggering of a signaling cascade initiated by ceramide synthesis has been observed in doxorbicin-induced apoptosis in murine leukemia cells[20]. Ceramide-mediated signaling has also been shown to be involved in the CD95 signal transduction pathway[21]. However, doxorbicin-induced stimulation of ceramide synthesis was found at concentrations above therapeutic levels (>1 μg/ml). At these levels we did not detect induction of CD95-L mRNA expression. This may suggest that high concentrations of doxorbicin may bypass the requirement for CD95/CD95-L interaction in triggering death in leukemia cells[20]. However, at therapeutic concentrations of doxorbicin, apoptosis triggered via CD95/CD95-L interaction may be the predominant mechanism of drug-induced death. In addition to leukemia cells, induction of CD95-L expression following treatment with cytotoxic drugs is also observed in neuroblastoma and hepatocellular carcinoma cell lines (data not shown and M. Müller *et al.*, manuscript submitted). The molecular pathway that mediates CD95-L expression by cytotoxic drugs may involve accumulation of wild-type p53 and transcriptional activity of the p53 protein (data not shown). Taken together, our data provide a basis for the molecular understanding of sensitivity and resistance of leukemias by demonstrating a central role for the CD95 system in drug-induced apoptosis. The cytotoxic action of drugs used in chemotherapy of leukemias and solid tumors may involve several levels of interference with apoptosis pathways that include triggering of CD95/CD95-L interaction (autocrine suicide), activation of several signal transduction pathways (ceramide) and activation of effector molecules such as ICE-like proteases[22]. Because CD95 and CD95-L are expressed on a variety of tumors and tissues *in vivo* our data may have broad implications for tumor therapy and for side effects of cytotoxic drugs on normal tissues.

Methods

Cell lines and culture conditions. CEM cells were grown in RPMI 1640 (Gibco BRL, Eggenstein, Germany) containing 10% FCS (Conco, Wiesbaden, Germany), 10 mM HEPES, pH 7.3 (Biochrom, Berlin, Germany), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco) and 2 mM L-glutamine (Biochrom). CEM^R, a variant of CEM resistant to anti-CD95 was generated by continuous culture in medium containing anti-APO-1 (anti-CD95) (IgG3, 1 μg/ml) for more than 2 months. For experiments CEM^R cells were washed twice with RPMI 1640 medium and cultured in medium for 24 h without anti-CD95. Likewise Jurkat^R cells were generated by continuous culture in medium containing anti-CD95 (IgG3, 1 μg/ml) for 6 months.

Drugs. Doxorubicin (Farmitalia, Milano, Italy) and methotrexate (Lederle, Wolfratshausen, Germany) were provided as pure substances. Doxorubicin was freshly dissolved in sterile distilled water

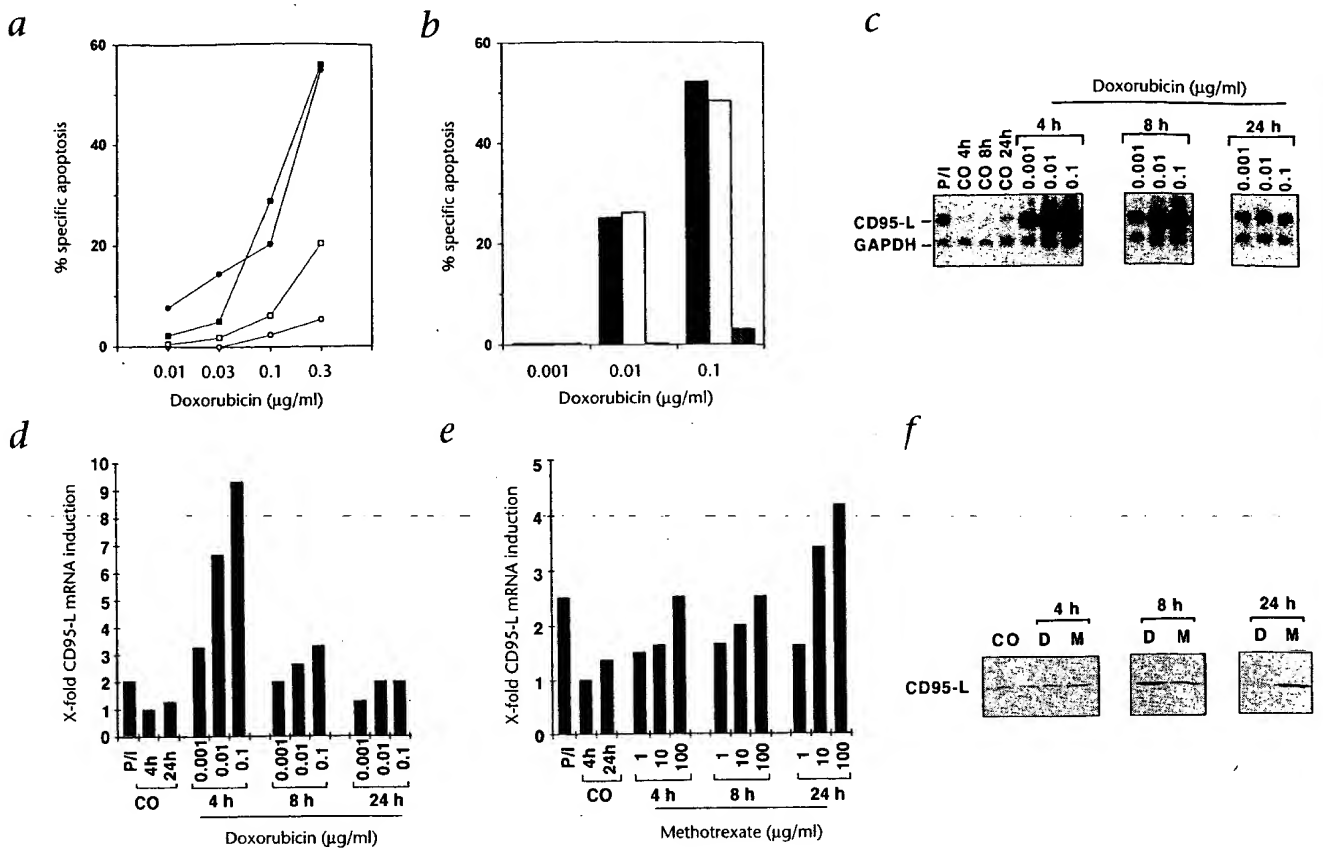

Fig. 2 Involvement of CD95/CD95-L in drug-induced apoptosis. *a*, Doxorubicin-induced apoptosis in anti-CD95-sensitive and resistant (R) leukemia cells. CEM (closed dots) and CEM^R (open dots) cells, Jurkat (closed squares) and Jurkat^R (open squares) cells were incubated in 96-well plates (2×10^4 cells/well) with different concentrations of doxorbicin for 24 h. Apoptosis was determined by FSC/SSC. Data are given as the means of triplicates with a standard deviation of less than 10% in each case. A representative experiment of three experiments with similar results is shown. Controls using different concentrations of anti-CD95 were included in each experiment to prove anti-CD95 resistance of CEM^R and Jurkat^R cells at the time of experiment (data not shown). *b*, Inhibition of doxorbicin-induced apoptosis in CEM cells by F(ab')$_2$ anti-APO-1 (anti-CD95). CEM cells were incubated in 96-well plates (2×10^4 cells/well) with different concentrations of doxorbicin for 30 h in the presence of medium (black bars), F(ab')$_2$ FII23 (IgG3 control antibody) (white bars) or F(ab')$_2$ anti-APO-1 (blocking antibody) (grey bars). Cells were preincubated with F(ab')$_2$ fragments (1 µg/ml) for 30 minutes before addition of doxorbicin. Specific apoptosis was measured and calculated as described in Fig. 1. Data are given as the means of triplicates with a standard deviation of less than 10% in each case. Similar results were obtained in three independent experiments. *c*, Induction of CD95-L mRNA expression by doxorbicin and methotrexate. CEM cells were incubated with medium or doxorbicin at concentrations and time points indicated. CD95-L mRNA expression was determined by northern blot analysis. As positive control, CEM cells were incubated for 4 h with TPA (P, 2 ng/ml) and ionomycine (I, 2 µg/ml). *d* and *e*, CEM cells were incubated with medium, doxorbicin or methotrexate at concentrations and time points indicated. CD95-L mRNA expression was determined by quantitative PCR (*d*, *e*). *f*, Induction of CD95-L protein by doxorbicin and methotrexate. In *c*, CEM cells were incubated with 0.1 µg/ml doxorbicin (D) or 100 µg/ml methotrexate (M). At time points indicated the specific band representing the 30-kDa CD95-L protein was detected by western blot. For measurement of baseline levels of CD95-L, cells were cultured in medium (CO). Protein loading was controlled by Ponceau red staining.

and methotrexate was freshly dissolved in 0.01 M NaOH before each experiment to ensure constant quality of the preparations.

Determination of apoptosis. For quantitative determination of apoptosis, cells were lysed in Nicoletti buffer (0.1% sodium citrate plus 0.1% Triton X-100 containing propidium iodide 50 µg/ml) as described[23]. Propidium iodide-stained nuclei or FSC/SSC profile of nonfixed cells were analyzed by flow cytometry (FACScan, Becton Dickinson, Heidelberg, Germany)[23,24]. For analysis of DNA fragmentation, cell pellets were treated with 1.5 ml lysis buffer (10 mM Tris-HCl, 400 mM NaCl, 2 mM Na_2EDTA, pH 8.2, and 0.1 ml 10% SDS) and proteinase K (2 mg/ml) (Boehringer Mannheim, Germany) and dissolved in lysis buffer at 37 °C overnight. Protein was precipitated with 0.5 ml 5 M NaCl at room temperature (1 h) and centrifuged at 3300*g*. DNA in the supernatant was precipitated overnight with 2 volumes of dried ethanol at –20 °C. After centrifugation at 3300*g*, the pellet was dissolved in 99 µl of 10 mM Tris-HCl/0.2 mM Na_2EDTA (pH 7.2) and incubated with 1 µl RNase (0.5 mg/ml, Boehringer Mannheim) at 37 °C overnight followed by electrophoresis on a 1.7% agarose gel (30 V for 6 h). The agarose gel was stained with ethidium bromide, and the resulting DNA fragmentation pattern was revealed by UV illumination.

Preparation of F(ab')$_2$ fragments. F(ab')$_2$ fragments of anti-APO-1 and isotype-matched antibody FII23 (IgG3) were prepared as described[7].

Northern blot analysis. For northern blot analysis, mRNA was prepared using oligo dT cellulose, blotted onto nitrocellulose filters and probed with a 500-base pair (bp) CD95-L fragment as described[25]. A cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control.

Quantitative RT-PCR. Expression levels of CD95-L mRNA by quantitative RT-PCR were determined as described[25], using a competitor fragment of CD95-L sequence of higher molecular size (550 bp) coamplified with the wild-type CD95-L fragment (500 bp). Constitutive levels of CD95-L mRNA were arbitrarily given a value of 1 (CO 4 h: 300 fg CD95-L mRNA/25 ng mRNA).

Western blot analysis of CD95-L. For western blotting, proteins obtained from whole-cell lysates were separated by 15% PAGE (ref. 14) and transferred to ECL membranes (Amersham, Braunschweig, Germany). Detection of CD95-L was performed using a rabbit polyclonal antibody that recognizes human CD95-L peptide (Santa Cruz Biotechnology, Santa Cruz, California) and the ECL system (Amersham). Equal protein loading was proven by Ponceau red treatment of membranes. Using this polyclonal antibody no 30-kDa CD95-L protein is detected in cells that are negative by RT-PCR.

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p53-dependent apoptosis suppresses radiation-induced teratogenesis

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About half of human conceptions are estimated not to be implanted in the uterus, resulting in unrecognizable spontaneous abortions[1,2], and about 5% of human births have a recognizable malformation[1,3]. In order to find clues to the mechanisms of malformation and abortion, we compared the incidences of radiation-induced malformations and abortions in *p53* null (*p53*$^{-/-}$) and wild-type (*p53*$^{+/+}$) mice. After X-irradiation with 2 Gy on day 9.5 of gestation, *p53*$^{-/-}$ mice showed a 70% incidence of anomalies and a 7% incidence of deaths, whereas *p53*$^{+/+}$ mice had a 20% incidence of anomalies and a 60% incidence of deaths. Similar results were obtained after irradiation on day 3.5 of gestation. This reciprocal relationship of radiosensitivity to anomalies and to embryonic or fetal lethality supports the notion that embryonic or fetal tissues have a *p53*-dependent "guardian" of the tissue[4] that aborts cells bearing radiation-induced teratogenic DNA damage. In fact, after X-irradiation, the number of cells with apoptotic DNA fragments was greatly increased in tissues of the *p53*$^{+/+}$ fetuses but not in those of the *p53*$^{-/-}$ fetuses.